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Short Communication

Infection characteristics of rabies virus variants with deletion or insertion in the pseudogene sequence

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We investigated the infection characteristics of recombinant rabies virus variants modified in the pseudogene sequence. Infection of neuronal cell lines by the SAD W9 and SAD V* variants (respectively with deletion or insertion in this sequence) showed no significant differences as compared to the parental strain, the attenuated strain SAD B19, in infection characteristics such as number of infected cells or viral yield. The inoculation of mice by these variants resulted in similar infection patterns and pathogenicity. Stereotaxic inoculation of the different variants into the rat striatum showed that deletion or insertion did not affect the axonal virus spread, nor did insertion of a complete additional transcription unit, that could be expressed in the areas connected to the inoculation site. These results show that the pseudogene sequence is not involved in viral spread and pathogenicity and confirm the availability of this domain for targeting and expression of foreign genes into neurons.

Keywords: reverse genetics; axonal transport; brain; pathogenesis

Introduction

Rabies virus is a highly neurotropic agent which is thought to induce its clinical picture through brain dysfunction rather than neuronal destruction (for a review, see Tsiang, 1993). Until now, most of the involvement of the different rabies proteins in pathogenesis has been poorly understood. Recently, it has been possible by a reverse genetic approach, to perform genetic manipulation of non-segmented negative-strand RNA viruses (for a review, see Conzelmann, 1996) and to obtain infectious rabies viruses from cloned cDNA (Schnell *et al*, 1994). The first manipulated rabies virus variants to be described were two constructs modified in the so-called 'pseudogene Ψ region' (which corresponds to the non-transcribed 3′ sequence of the G gene, Ravkov *et al*, 1995) from the SAD B19 strain, an attenuated live vaccine virus which is however able to kill rodents after intracerebral and even oral administration (Artois *et al*, 1992). One variant (SAD W9) lacked this 0.4 kb sequence whereas in the other (SAD V*) a functional extra cistron border was introduced (Schnell *et al*, 1994) (Figure 1). Both of these variants have been shown to infect fibroblastic cell lines comparable to the parental strain SAD B19 (Schnell *et al*, 1994). More recently, insertion of a complete additional transcription unit encoding bacterial chloramphenicol acetyl transferase (CAT) has been described, either by replacement of the pseudogene sequence (SAD VCAT), or by addition to the pseudogene sequence (SAD X-CAT) (Figure 1). Both of these variants were shown to express the foreign gene in a stable manner after infection of fibroblastic cell lines (Mebatsion *et al*, 1996). The finding that the pseudogene sequence is dispensable for virus propagation in fibroblast cells was compelling, since rabies viruses analysed so far have conserved this sequence, and non-essential sequences are usually eliminated rapidly from RNA virus genomes. Although not required for basic virus replication, the pseudogene sequences might therefore have a so far not appreciated function in the infected host, for example in determining spread and/or pathogenesis.

To evaluate the role of the Ψ sequence in the infection of the main target of rabies virus, i.e. the neuron, we compared the behaviour of the different variants described above to the parental strain SAD B19 in the *in vitro* infection of neuroblastoma cell lines and *in vivo* infection of mouse brain. We additionally evaluated the

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axonal transport of these different variants in the rat brain using a *in vivo* model of stereotaxic inoculation as previously described (Gillet *et al*, 1986; Ceccaldi *et al*, 1989).

Infection of neuronal cell lines by the different variants

Study of the infection of neuronal cell cultures was performed by plating neuro 2A cells in 96-wells microplates (10^3/well) 24 h before viral infection (for 1 h at 37°C) with one of the different variants, at a multiplicity of infection (m.o.i.) of 1 p.f.u./cell. Experiments were performed in Dulbecco's medium supplemented with gentamycine, glutamine, and foetal calf serum (10%). Three days later, cultures were processed for rabies nucleocapsid detection by fixation in −20°C acetone and incubation with a polyclonal rabbit anti-rabies nucleocapsid serum coupled with fluorescein (Diagnostics Pasteur, France) (Atanasiu *et al*, 1974). Visualization of rabies nucleocapsid inclusions was performed using a Leica (Germany) DMRB fluorescence microscope equipped with a video camera, by counting the number of fluorescent foci in three different fields of each well (12 wells per variant). On day 3 post-infection (p.i.), infection of cells resulted in a similar pattern of rabies inclusions morphology and distribution throughout the cytoplasm for the three variants (data not shown) with a mean number of fluorescent foci per field of 6.889 (s.d. ± 0.687), 5.611 (s.d. ± 0.993) and 5.5 (s.d. ± 0.732) for SAD L16, V* and W9 respectively (36 fields observed per variant). We investigated the viral yield of the three variants by culturing neuro 2A cells on culture flasks according to conditions described above. Viral yield was evaluated by the plaque titration assay (Smith *et al*, 1977). As shown in Table 1, no significant difference was noted for the three variants on days 4 and 6 p.i.

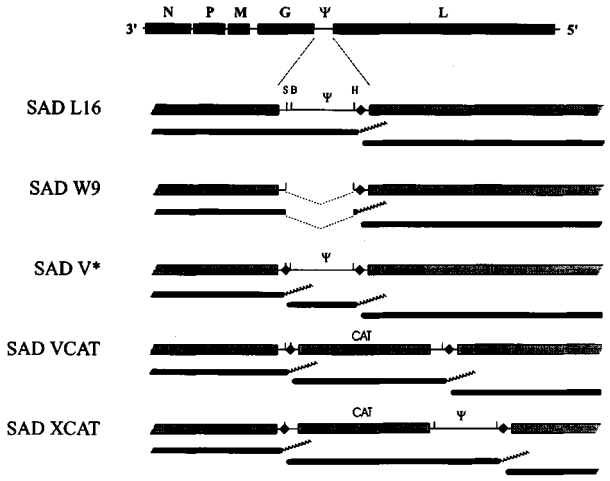

Figure 1 Genome organisation and transcription of rabies virus SAD B19 variants modified in the pseudogene sequence (Ψ). Open reading frames are shown as filled boxes, gene borders (transcription stop/polyadenylation, intergenic sequence, restart) as rhomboid symbols (◆), and mRNA as black bars. S, B, and H represent restriction sites used for construction of cDNA clones (*Sty*I, *Bam*HI, *Hind*III, respectively).

Pathogenicity and viral infection of mice by the different variants

To evaluate the *in vivo* behaviour of the variants, 20 gr. male mice (OF1, IFFA-Credo, France) were inoculated intra-cerebrally (3000 p.f.u./mouse, volume: 0.03 ml) with each of the following variants: SAD L16, W9, V*, XCAT and VCAT (9 or 10 mice/variant). All the mice succumbed and study of death rate revealed no significant difference between the different variants, with values comprised between days 8 and 11 p.i. (L16: 9.7 s.d. ± 0.94; W9: 9 s.d. ± 1.41; V*: 8 s.d. ± 1.15; VCAT: 10.11 s.d. ± 0.33; XCAT: 8.5 s.d. ± 0.52). Brain infection was assessed by detecting viral nucleocapsid inclusions on cryostat sections (20 μm thick) at the death of the animals, with a rabbit anti-nucleocapsid FITC conjugate as previously described (Ceccaldi *et al*, 1989). As shown in Figure 2, no difference was seen between the different variants concerning both the brain localization of viral inclusions and intensity of infection, throughout the whole brain.

Viral spread of the different variants

To further analyse the virus spread of the variants, we performed stereotaxic inoculation of the different virus variants into the rat striatum. This assay allows the study of early spread and axonal transport of rabies virus to the striatum-connected brain areas (Gillet *et al*, 1986; Ceccaldi *et al*, 1989). Briefly, male Wistar rats (120–150 gr.; CER Janvier, France) were inoculated bilaterally after deep anesthaesia (Imalgene-Vetranquil) in the striatum with 2 μl of viral suspension (10^4 p.f.u./animal) (12 rats/variant), as previously described (Gillet *et al*, 1986). On days 3, 4 and 8 p.i., rats were euthanasied, brains were rapidly removed and frozen in liquid nitrogen and processed as described above for immunolocalization of viral inclusions. Results in Figure 3 show that on day 3 p.i., viral inclusions are detected according to a similar pattern in the brains infected by SAD L16, V* and W9 variants. At this stage, viral inclusions can be detected not only in the inoculation site (striatum) but also in the cortex, thalamus, hippocampus, septum, substantia

Table 1 Viral yield of SAD L16, V* and W9, measured by plaque titration assay, on neuronal cell cultures (neuro 2A) on days 4 and 6 post infection. Infection was performed as described in text. Results expressed in p.f.u./ml.

Viral yield	*SAD L16*	*Sad V**	*SAD W9*
Day 4 post infection	1.7×10^6	5×10^6	1.6×10^6
Day 6 post infection	9.9×10^6	1.15×10^7	4.8×10^6

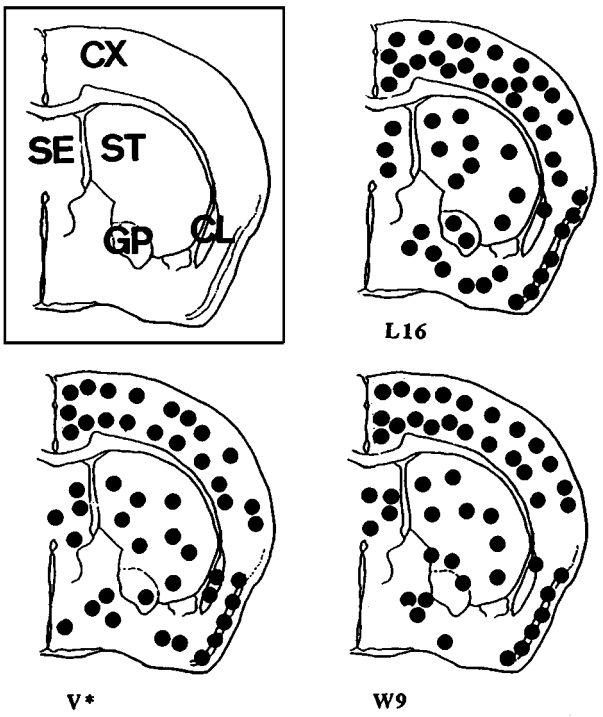

Figure 2 Schematic localization of viral inclusions detected by direct immunofluorescence (with an anti-rabies nucleocapsid fluorescent conjugate, on cryostat sections) in mouse brain for the three different variants (SAD L16, V* and W9), on day 10 post infection, after an intra-cerebral inoculation of 10^3 p.f.u./mouse. Each circle represents one or one group of fluorescent foci. Level A 4,450 of the Mouse stereotaxic atlas from Lehman (1974). C: Cortex; ST: Striatum; SE: Septum; GP: Globus Pallidus; CL: Claustrum.

nigra (pars compacta) and amygdala, whereas no inclusions are detected in the pars reticulata of the substantia nigra and in the cerebellum. On day 4 p.i., infection spreads in a similar manner for the three variants (data not shown). On day 8 p.i., the first clinical signs are observed for the three variants (pilo-erection, cachexia, paralysis), accompanied by a widespread infection of the brain (data not shown).

Targeted delivery of a complete additional transcription unit

Study of the SAD XCAT variant gave similar results concerning viral transport after stereotaxic inoculation, when detected by immunofluorescence on cryostat sections (data not shown). Moreover, we performed an analysis of the CAT expression in the different brain areas of the rats infected by sterotaxic inoculation into the striatum. Briefly, the rats were infected as described above (10^4 p.f.u./striatum) and on day 4 p.i., animals were euthanasied, and brain areas rapidly removed according to brain microdissection techniques (Cuello, 1983), then frozen in liquid nitrogen and homogenized to be processed for a CAT- ELISA assay (Boehringer Mannheim, Germany). As shown in Figure 4, in infected animals CAT expression is detected in the inoculation site (striatum) of infected animals but also in the anatomically connected areas such as thalamus, cortex and substantia nigra. Cerebella did not show any significant CAT expression at this stage, compared to the uninfected control.

Taken together, the present data indicate that genetic manipulations of the pseudogene region,

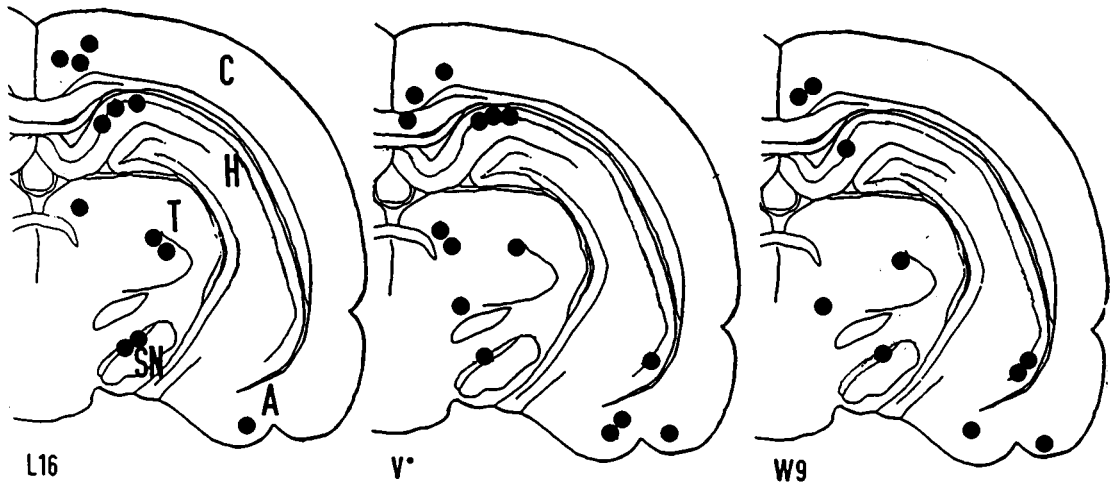

Figure 3 Schematic localization of viral inclusions detected by direct immunofluorescence (with an anti-rabies nucleocapsid fluorescent conjugate, on cryostat sections) in the rat brain after stereotaxic inoculation of 10^4 p.f.u. in right and left striatum, for the SAD L16, V* and W9 variants. Day 3 post infection. C: Cortex; SN: Substantia Nigra, H: Hippocampus; T: Thalamus; A: Amygdala. Each circle represents one or one group of fluorescent foci. According to the level A2420 of the Rat Brain stereotaxic atlas from König and Klippel (1974).

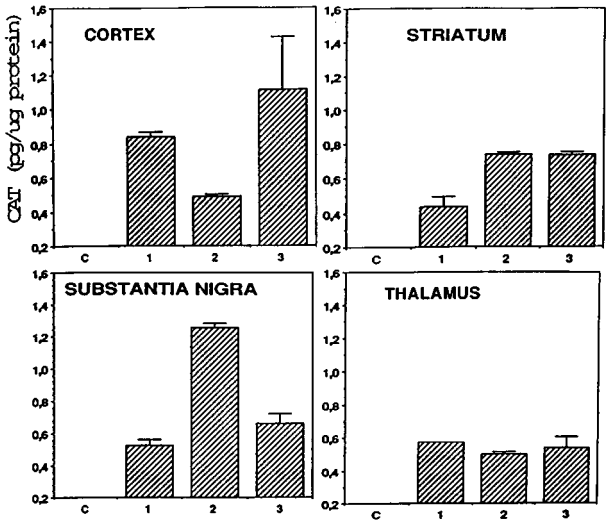

Figure 4 CAT expression in different rat brain areas after stereotaxic inoculation of the SAD XCAT variant in the striatum. Brain sequences were dissected on day 4 post-infection as described in text; CAT expression was assessed with a CAT ELISA assay; results are expressed in pg/μg protein for uninfected control (C) and for three different infected animals (1; 2 and 3).

including the introduction of additional sequences and deletions, do not result in obvious alterations of the infection characteristics of rabies virus either on neuronal cell cultures or in mouse and rat models. It has been shown previously that this region, thought to be a remnant protein gene (Tordo *et al*, 1986), could be modified by transcriptional unlinkage or deletion without interfering with the ability to replicate and propagate in fibroblastic cell cultures (Schnell *et al*, 1994). This report demonstrates that the presence of the pseudogene sequence has no influence on the infection behaviour of rabies virus in its main target, i.e. the neuron. The modification of the pseudogene sequence did not affect the brain distribution of viral infection of mice nor the pathogenicity, showing that the pseudogene sequence is not involved in the behaviour of rabies virus after experimental infection of animals, at least by the methods used in these experiments.

Experiments with the rat stereotaxic inoculation model demonstrate that this sequence did not influence the rabies virus spread via the axonal transport, since in the early steps of infection viral inclusions are only detected in the striatum-connected areas, whatever the variant used. The fact that on day 3 p.i. only the pars compacta of the substantia nigra is infected whereas the pars reticulata is spared, suggests that the modified variants spread via the retrograde axonal transport in the first steps of infection, since the pars compacta is afferent to the striatum and the pars reticulata is efferent (Robertson and Travers, 1975). This retrograde axonal transport is in agreement with the spread of the fixed virulent strain CVS (Gillet *et al*, 1986; Ceccaldi *et al*, 1989). Addition of a complete transcriptional unit is also unable to modify the rabies virus axonal transport (SAD XCAT variant) in rats and the pathogenicity in mice (SAD VCAT and XCAT variants). The efficiency of axonal transport for the spread of this variant can also be demonstrated by the level of CAT expression in the striatum-connected areas such as cortex, hippocampus, thalamus and substantia nigra, whereas no CAT expression is detected in the cerebellum which is not directly connected to the inoculation site (Hebel and Stromberg, 1986). According to the behaviour of the SAD XCAT variant we conclude that recombinant rabies virus may be used as a vector for expression of foreign genes in specific brain areas that can be reached via retrograde axonal transport. In a previous report, Mebatsion *et al*. (1996) had shown the ability of rabies virus to express foreign genes in a stable manner in fibroblastic cell lines and in mouse brains. The present results show that a functional foreign gene can be specifically targeted on brain sequences using the rabies virus spread via axonal transport (Bijlenga and Heaney, 1978; Tsiang, 1979; Kucera *et al*, 1985; Gillet *et al*, 1986; Ceccaldi *et al*, 1989) without apparently changing the neurotropic characteristics of the virus (Tsiang *et al*, 1983).

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